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1***Gigaspora margarita* with and without its endobacterium shows adaptive responses to**
2**oxidative stress**

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34

35Abstract

36Arbuscular mycorrhizal (AM) fungi experience oxidative stress during the plant-fungal interaction,
37due to endogenous reactive oxygen species (ROS) produced by fungal metabolism and exogenous
38ROS produced by plant cells. Here, we examine the responses to H₂O₂ in *Gigaspora margarita*, an
39AM fungus containing the endobacterial symbiont *Candidatus Glomeribacter gigasporarum*
40(*CaGg*). Previous studies revealed that *G. margarita* with its endobacterium produces more ATP
41and has higher respiratory activity compared to a cured line that lacks the endobacterium. This
42higher bioenergetic potential leads to higher production of ROS, and to a higher ROS-detoxifying
43capacity, suggesting a direct or indirect role of the endobacterium in modulating fungal anti-oxidant
44responses. To test the hypothesis that the fungal-endobacterial symbiosis may enhance the fitness of
45the AM fungus in the presence of oxidative stress, we treated the fungus with a sublethal
46concentration of H₂O₂ and performed RNA-seq analysis. Our results demonstrate that: i)
47irrespective of the endobacterium presence, *G. margarita* faces oxidative stress by activating
48multiple metabolic processes (methionine oxidation, sulfur uptake, the pentose phosphate pathway,
49activation of ROS-scavenger genes), ii) in the presence of its endobacterium, *G. margarita*
50upregulates some metabolic pathways, like chromatin status modifications and iron metabolism; iii)
51contrary to our hypothesis, the cured line responds to H₂O₂ by activating the transcription of specific
52ROS scavengers. We confirmed the RNA-seq findings by measuring the glutathione and ascorbate
53content, which was the same in both lines after H₂O₂ treatment. We conclude that both fungal lines
54may face oxidative stress, but they activate alternative strategies.

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66Key words

67Arbuscular mycorrhizal fungi; Endobacteria; RNA-seq; Oxidative stress; Reactive oxygen species

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69Introduction

70As obligate biotrophs that colonize the roots of most land plants and enhance the host's ability to
71acquire nutrients, arbuscular mycorrhizal (AM) fungi have important roles among the plant
72microbiota. Some AM fungi possess obligate endobacteria inside their cytoplasm, giving rise to an
73endosymbiosis which is not essential for the fungal host (Bonfante and Desirò 2017; Olsson et al.
742017). The genome sequences of rod- and coccoid-shaped endobacteria revealed that both have a
75reduced genome and a strong dependence on the fungal host (Ghignone et al. 2012; Torres-Cortés et
76al. 2015). The rod-shaped bacterium, *Candidatus Glomeribacter gigasporarum* (CaGg), has been
77more deeply investigated: as an obligate endosymbiont of the AM fungus *Gigaspora margarita*,
78CaGg relies on its fungal host for nutrition, thus representing a physiological cost to the fungus.
79However, the bacterial population has been maintained in the fungus over many generations in an
80association that is believed to be as ancient as the mycorrhizal symbiosis (Mondo et al. 2012;
81Bonfante and Desirò 2017).

82The benefits that the endobacteria provide to the fungal partner remain largely unknown, although
83recent studies on other fungal-bacterial interactions have given some hints (Uehling et al. 2017; Li
84et al. 2017). In a previous study, we combined next-generation sequencing, molecular biology, and
85cell physiology analyses to compare *G. margarita* containing CaGg (B+ line) with a “cured” line of
86the fungus that lacks CaGg (B- line) obtained under laboratory conditions (Lumini et al. 2007). We
87demonstrated that CaGg can enhance its host's bioenergetic potential in terms of ATP production,
88by increasing mitochondrial respiration rates in spores germinated in H₂O (Salvioli et al. 2016). In a
89comparison between B+ and B- germinating spores, RNA-seq data revealed that the B+ line showed
90overall higher expression of genes involved in the response to reactive oxygen species (ROS)
91(Salvioli et al. 2016), including several ROS-scavenging enzymes. The results pointed to a
92relationship between cellular respiration and superoxide radical production (as a direct byproduct of
93the electron transport chain). Taking in account the results discussed above, we argued that CaGg
94may have a protective effect on *G. margarita*, making the fungal host more efficient in detoxifying
95endogenous ROS. Consistent with this, proteomic data confirmed that the B- spores produced less
96thioredoxin and other ROS-detoxifying proteins, and showed signs of oxidative damage (Vannini et
97al. 2016).

98ROS cause detrimental chemical changes in proteins, lipids, polysaccharides, DNA, RNA, and even
99in small metabolites (Winterbourn 2008). Oxidative stresses can produce diverse responses in
100filamentous fungi, ranging from mitochondria-mediated ageing and programmed cell death
101(Osiewacz 2011) to signaling that may regulate mycelial development and sexual reproduction
102(Breitenbach et al. 2015). Oxidative stresses can originate from endogenous or exogenous sources

103and may play different roles. On the one hand, H₂O₂ may act as a signal in response to injury in
104fungi such as *Trichoderma* and *Rhizophagus irregularis*, suggesting that fungi share a defense
105mechanism based on ROS-related molecules, as is common in plants and animals (Hernández-
106Oñate et al. 2012; Fester and Hause 2005). On the other hand, environmental stimuli can lead to
107oxidative stress; for example, a recent study reported that the glutathione peroxidase redox system
108is directly involved in the resistance of *Alternaria alternata* to the fungicides fludioxonil and
109vinclozolin (Yang et al. 2016). Current information on how AM fungi respond to exogenous
110oxidative stress is restricted to the intraradical stages of fungal development (Belmondo et al. 2016;
111Lanfranco et al. 2005), and define a scenario in which host plant-produced ROS are part of a fine-
112tuned signaling pathway necessary for fungal development. Similar experiments have described
113how *Beauveria bassiana* (an entomopathogenic fungus) responds to insect-produced ROS (Zhang et
114al. 2015).

115Few studies have examined the response of AM fungi to induced oxidative stress when the AM
116fungi are living in the soil as extraradical hyphae. A recent investigation (Tamayo et al. 2016)
117revealed that five *R. irregularis* glutaredoxin genes respond to H₂O₂ and may have a role in iron
118homeostasis. By using an omics approach, we wanted to test the hypothesis that *G. margarita* can
119respond to exogenous oxidative stress by activating specific anti-oxidants and that the presence of
120its endobacterium enhances the fungal response to oxidative stress. Preliminary results (Salvioli et
121al. 2016) suggested that both the bacterial and fungal symbionts perceived the oxidative stress.
122Here, we set up an RNA-seq approach to describe the response of an AM fungus to H₂O₂ treatment,
123validating the transcriptomic data with antioxidant measurements. We demonstrate that *G.*
124*margarita* with its endobacterium is equipped to respond to environmental oxidative stresses, but
125also the cured line successfully responds to H₂O₂ with the expression of genes involved in specific
126ROS- related pathways.

127

128Materials and Methods

129

130Spore production

131*Gigaspora margarita* Becker and Hall (isolate BEG 34, deposited at the European Bank of
132Glomeromycota) was used in this study. This strain naturally contains endobacteria (Bianciotto et
133al. 1996) and is identified here as the B+ line. The cured line, i.e. without the endobacterium (B-
134line), was obtained from the B+ line as described in Lumini et al. 2007. To maintain and propagate
135both lines, we used pot cultures with white clover (*Trifolium repens*) as a trap plant, inoculating

136100–150 spores for each pot. Second-generation spores were produced within 3 months, and were
137then collected by the wet sieving technique (Gerdemann and Nicolson 1963).

138

139Treatment with hydrogen peroxide

140

1416000 B+ and 6000 B- spores were surface sterilized with a solution containing 3% P/V Chloramine
142T and 0.03% P/V streptomycin sulphate, and subsequently washed with sterile water three times for
14310 minutes each. The sterilized spores were divided into batches of 100 (each batch corresponding
144to one biological replicate), placed in 10 wells of 12 multi-well plates, and incubated for 3 days at
14530°C in the dark, for germination pre-conditioning. After this step, the B+ and B- spores of half of
146the wells were treated with 0.3 mM H₂O₂, previously identified as the highest concentration at
147which the fungus could successfully germinate (Salvioli et al. 2016). Sterile water was replaced in
148the other half of the wells. After 3 hours of incubation at 30°C in the dark, 3 biological replicates
149(300 spores) from each condition were collected and frozen in liquid nitrogen for RNA extraction.

150

151RNA seq experiments

152RNA extraction, library preparation, and sequencing were performed according to previously
153described protocols (Salvioli et al. 2016). Total RNA was extracted using the RNeasy Microarray
154Tissue Mini Kit (Qiagen, Germany). To measure the concentration and quality of the extracted
155samples, we used the Nanodrop1000 (Thermo Scientific, Wilmington, NC, USA) and RNA integrity
156was assessed with the Bioanalyzer instrument (Agilent Technologies, Santa Clara, CA, USA). Three
157Illumina single-end libraries, corresponding to three biological replicates, were generated for each
158condition (B+ control spores, B- control spores, B+ treated spores, and B- treated spores, for a total
159of 12 libraries). Illumina HiScanSQ sequencing (50-bp single-end reads) was performed at the
160Genomic facility of the HuGeF (<http://www.hugef-torino.org/site/index.php?id=102&t=articolo>)
161and of DBios, University of Torino [http://www.dbios.unito.it/do/home.pl/View?](http://www.dbios.unito.it/do/home.pl/View?doc=servizi_esterno/analisi_genomica.html)
162doc=servizi_esterno/analisi_genomica.html) following ENCODE project standard protocols
163([http://encodeproject.org/ENCODE/protocols/dataStandards/ENCODE_RNAseq_Standards_V1.0.p](http://encodeproject.org/ENCODE/protocols/dataStandards/ENCODE_RNAseq_Standards_V1.0.pdf)
164df). Libraries were processed with Trimmomatic V.0.36 (settings: LEADING:10, TRAILING:10,
165SLIDINGWINDOW:4:25, MINLEN:25, Q32, ILLUMINACLIP:TruSeq3-PE.fa:2:30:10) to remove
166low-quality reads and Illumina adapters (Bolger et al. 2014). Raw reads were then mapped to *G.*
167*margarita* assembled transcripts (Salvioli et al. 2016) using Bowtie2 (Langmead and Salzberg
1682012).

169

170Differential expression analysis

171DESeq2 1.12.4 Bioconductor package was used for the identification of differentially expressed
172genes (DEGs), setting local fit and betaPrior parameters to TRUE. Independent filtering was
173enabled (Anders and Huber 2010; Love et al. 2014). A threshold of 0.05 was applied to false
174discovery rates (FDR) to filter for significant DEGs. An additional filter was applied on log₂ fold
175changes, and only genes with a |log₂ FC| higher than 1.5 were selected for each comparison. The
176following 4 comparisons were investigated: treated spores vs control spores (regardless of the
177endobacterial presence); B+ treated spores vs B+ control spores; B- treated spores vs B- control
178spores; and B+ treated spores vs B- treated spores. Gene Ontology (GO) enrichment for each
179comparison was performed with the AgriGO web platform (<http://bioinfo.cau.edu.cn/agriGO/>) and
180visualized with Gplot v.1.0.2 (Walter et al. 2015). BLAST searches were performed with the
181BLAST+ suite (Camacho et al. 2009).

182

183Extraction and analysis of ascorbate and glutathione

184A new set of B+ and B- control and treated spores was obtained as described in the “Treatment with
185hydrogen peroxide” section. For each condition ascorbate and glutathione were measured in
186independent experiments by using three biological replicates of 1000 spores. The material was
187ground in liquid nitrogen and homogenized with 1 mL of cold 5% meta-phosphoric acid at 4°C in a
188porcelain mortar. The homogenate was centrifuged at 20,000 g for 15 min at 4°C, and the
189supernatant was collected for analysis of glutathione and ascorbate (or its analogues) according to
190de Pinto et al. (1999).

191The dipyrldyl assay was used for the measurement of all the ascorbate-like reductants (Spickett et
192al. 2000). Briefly, total content of ascorbate or its analogues was determined after reduction of the
193oxidized forms (DHA) with DTT, and the concentration of DHA was estimated from the difference
194between total pool of ascorbate and its analogues (ASC+DHA) and the reduced forms (ASC). The
195reaction mixture for ASC+DHA determination contained a 0.1 ml aliquot of the supernatant, 0.25
196ml of 150 mM phosphate buffer (pH 7.4) containing 5 mM EDTA, and 0.05 ml of 10 mM DTT.
197After incubation for 10 min at room temperature, 0.05 ml of 0.5% N-ethylmaleimide was added to
198remove excess DTT. ASC was determined in a similar reaction mixture except that 0.1 ml H₂O was
199added rather than DTT and N-ethylmaleimide. Colour was developed in both reaction mixtures after
200addition of the following reagents: 0.2 ml of 10% trichloroacetic acid, 0.2 ml of 44% ortho-
201phosphoric acid, 0.2 ml of 4% α,α'-dipyridyl in 70% ethanol and 0.3% (w/v) FeCl₃. After
202vortexing, the mixture was incubated at 40°C for 40 min and the A₅₂₅ was read. A standard curve
203was developed based on ascorbate in the range 0-50 µg ml⁻¹.

204The glutathione pool was assayed utilizing 0.4 ml aliquots of supernatant neutralized with 0.6 ml of
2050.5 M phosphate buffer pH 7.5. For glutathione disulphide (GSSG) assay, the reduced form of glu-
206tathione (GSH) was masked adding to the neutralized supernatant 20 µl of 2-vinylpyridine, whereas
20720 µl H₂O were added in the aliquots utilized for total glutathione pool assay. Tubes were mixed un-
208til an emulsion was formed. Glutathione content was measured in a 1 ml reaction mixture contain-
209ing 0.2 mM NADPH, 100 mM phosphate buffer pH 7.5, 5 mM EDTA, 0.6 mM 5,5'-dithiobis(2-ni-
210trobenzoic acid) and 0.1 ml of sample obtained as described above. The reaction was started by
211adding 3 units of glutathione reductase and was monitored by measuring the change in absorbance
212at 412 nm for 1 min. GSH was estimated as the difference between the amount of total glutathione
213and that of GSSG. A standard curve in the range 0-30 µM ml⁻¹ GSSG was prepared. Ascorbate and
214glutathione data were expressed as the average of three independent experiments. After checking for
215normality and homoscedasticity with Shapiro-Wilk and Levene's mean tests, respectively, the differ-
216ences between groups were assessed by using one-way analysis of variance (ANOVA) with a post-
217hoc Tukey's test, assuming $P < 0.05$ as statistically significant. The statistical analyses were per-
218formed using Sigma Plot software 12.0 (Systat Software, Inc., CA, USA).

219

220

221Results

222A previous study assessed the tolerance of the B+ and B- lines of *G. margarita* to a range of
223concentrations of hydrogen peroxide (Salvioli et al. 2016) from 0.2 to 2 mM. The study identified
2240.3 mM H₂O₂ as the highest concentration at which the fungus could successfully germinate. To
225describe the mechanisms that allow *G. margarita* to face exogenous oxidative stress, and to
226determine whether the presence of *CaGg* affects these dynamics, we selected the 0.3 mM H₂O₂
227concentration to treat B+ and B- germinating spores for the RNA-seq experiment.

228

229RNA-seq quantitative data: the overall response of *G. margarita* to hydrogen peroxide

230To examine the response to H₂O₂ on the transcriptional level, we first conducted transcriptome
231analysis by RNA-seq. To this end, we generated twelve Illumina single-end libraries, which yielded
232~10 to 17 million filtered reads (Table S1), giving a ~93% overall mapping rate on the *G. margarita*
233reference transcriptome (Salvioli et al. 2016). For each set of differentially expressed genes (DEGs),
234we applied a ± 1.5 log₂ fold change filter, and a total of 12527 genes among all the considered
235comparisons (of the 35029 currently predicted *G. margarita* genes) passed the filter. DEGs for each
236comparison are listed in Table S2, while Table S3 shows only the most up- or down- regulated
237genes.

To examine the effect of the endobacterium (B+ and B-) and the treatment (with and without H₂O₂) in shaping the fungal transcriptome, we performed Principal Component Analysis (PCA) and Variance Partitioning Analysis (VPA) on the whole dataset of normalized read counts (Fig. 1a and b, respectively). PCA analysis highlighted a strong differentiation between the control and treated samples (PC1, H₂O₂ treatment), but the separation between the data for the B+ and B- lines (PC2, endobacterial presence) was lower in both control and treated conditions. The lowest degree of separation was observed between the B+ and B- treated lines. Indeed, the direct comparison between the treated B+ fungus versus the treated B- fungus showed the fewest DEGs among all the comparisons (2423). The distance between the control and treated B- fungal samples was lower than the distance between the control and treated B+ fungal samples. This observation was consistent with the number of DEGs from the two comparisons (5543 vs 8291, respectively). VPA revealed the treatment with H₂O₂ as the factor mostly influencing the overall variability (~65%), while the presence/absence of the bacterium explained ~6% of the overall variability (Fig.1 b). These results confirm the PCA results, where the PC1 axis represents 72% of the variance vs the 18% represented by the PC2 axis.

Since the H₂O₂ treatment seemed to be the driving parameter, all the control fungal samples (B+ control and B- control) were compared with the treated samples (B+ treated and B- treated), irrespective of the presence of *CaGg*. This comparison identified 5458 DEGs, corresponding to about 15% of the *G. margarita* transcriptome (2398 up-regulated vs 3060 down-regulated genes).

257

258 **Hydrogen peroxide treatment: identification of the main Gene Ontology categories**

The results of GO enrichment analysis on this data set are shown in Fig 2. Among the enriched categories, the pentose-phosphate shunt, nicotinamide metabolic process, zinc ion transport, methionine metabolic process, and sulfur metabolic process categories were up-regulated overall in the treated samples.

The up-regulation of metabolic processes relative to sulfur and methionine is not surprising, since methionine containing proteins directly participate in defense against oxidative stress. Several types of ROS react with methionine, forming methionine sulfoxide, which is reduced by methionine sulfoxide reductase (Luo and Levine 2009). We previously demonstrated that under normal growth conditions, the B- line contains a higher quantity of carbonylated proteins (Vannini et al. 2016), which are considered a major hallmark of oxidative damage (Fedorova et al. 2014) and specifically involve methionine oxidation. The gene coding for methionine sulfoxide reductase showed a significant up-regulation in our data set upon H₂O₂ treatment (comp18281_c1, log₂ fold change: 12.69). Consistent with this, sulfur metabolism was among the enriched and up-regulated categories.

272The re-routing of carbon flux to the pentose-phosphate pathway has been described as a conserved
273response to oxidative stress (Ralser et al. 2007). In yeast, the pentose phosphate pathway is essential
274for sulfur assimilation, being the main reservoir of NADPH used by the cell for sulfite to sulfide
275conversion (Slekar et al. 1996). In *G. margarita* both the absence of *CaGg* (Salvioli et al. 2016;
276Vannini et al. 2016) and the H₂O₂ treatment seemed to induce the shift towards the pentose
277phosphate pathway. Taken in the whole, the GO enrichment analysis reveals that the H₂O₂ treatment
278pushes *G. margarita* to deeply change its transcriptome profile, shifting towards alternative
279metabolic pathways that are directly linked to mitigation of oxidative stress.

280To describe the oxidative stress response of *G. margarita* more in depth, DEGs from the treated vs
281control comparison (pooling results for B+ and B-) were further divided on the basis of their
282functional annotation, and we manually selected some genes known to be involved in ROS
283detoxification in fungi (Table 1). Among them, genes encoding glutathione peroxidase, glutathione
284reductase, and glutathione s-transferase, as well as a peroxiredoxin and a thioredoxin reductase
285were strongly up-regulated after the hydrogen peroxide treatment. Notwithstanding the central role
286that the glutathione cycle plays as a cellular redox buffer in yeasts and filamentous fungi,
287knowledge of its role and regulation in AM fungi is still limited (Benabdellah et al. 2009; Tamayo et
288al. 2016). Four glutaredoxin genes of *R. irregularis* have been characterized and demonstrated to be
289upregulated in the extraradical and intraradical mycelium following exposure to H₂O₂. The
290homologues of glutaredoxin *RiGRX1* (Benabdellah et al. 2009), *RiGRX4* and *RiGRX6* (Tamayo et
291al. 2016), were found in the *G. margarita* transcriptome (E-values: 3e-25, 9e-105 and 3e-63,
292respectively) and seemed to respond to H₂O₂. The homologue for *RiGRX5* was also found (E-value:
2932e-48), but its expression did not change significantly after H₂O₂ treatment. *G. margarita* catalase
294was also up-regulated upon treatment, but its expression level was not above the applied log₂ fold
295change threshold. Other players of the ROS-scavenging system, such as thioredoxin reductase, have
296already been identified as markers of *G. margarita* endogenous oxidative stress response (Salvioli
297et al. 2016; Vannini et al. 2016). Taken as a whole, these observations indicate that *G. margarita*
298modulates the expression levels of many redox enzymes in a way comparable to other filamentous
299fungi, as well as the model AM fungus *R. irregularis*. Moreover, given the number and the
300expression of the genes reported in Table 1, the glutathione/thioredoxin systems could be identified
301as key players in the oxidative stress response of *G. margarita*.

302To gather information on how *G. margarita* senses oxidative stress, we specifically searched for
303DEGs annotated as mitogen-activated protein kinases (MAPK) and transcription factors. This
304identified 14 DEGs encoding transcription factors and 160 encoding MAPKs (73 up-regulated vs 87
305down-regulated). Thirteen of these kinases possessed a PAS domain, which is common to bacteria,

fungi, and animals and is associated with osmotic and redox sensing (Taylor and Zhulin 1999; Moye-Rowley 2003). We then performed a BLAST search to find putative components of the High Osmolarity Glycerol Pathway (HOG), which is important for the response to oxidative stress in yeasts and filamentous fungi (Duran et al. 2010). Four top scoring *G. margarita* transcripts were differentially expressed upon H₂O₂ treatment, but without a clear expression pattern (Table S4). Given the high number of putative redox sensing elements whose expression is modulated by the treatment in a relatively short time (3h), we suggest that *G. margarita* evolved some specific mechanisms to perceive ROS also when growing outside the plant. Indeed, previous works demonstrated that AM fungi are exposed to H₂O₂ produced by the plant during the symbiotic phase, and that H₂O₂ concentrations could change the outcome of the symbiosis by being perceived as a signaling molecule (Kiirika et al. 2012; Arthikala et al. 2014; Belmondo et al. 2016).

The effect of *CaGg* on the response of *G. margarita* to hydrogen peroxide

PCA (Fig. 1a) revealed that the treatment with H₂O₂ reduced the differences between the B⁺ and the B⁻ fungal line. Since the B⁻ transcriptional profile seemed to be “triggered” by H₂O₂ becoming closer to the treated B⁺ profile, we analyzed the fungal B⁺ and B⁻ responses in more detail. The Venn diagram (Fig. 3) highlighted a subset of 1716 genes that were identified as DEGs in all the three comparisons (all treated vs all control, B⁺ treated vs B⁺ control, B⁻ treated vs B⁻ control). This subset can be referred to as the ‘core’ response of *G. margarita* to H₂O₂. Among this common gene core, the transcripts directly involved in ROS-related metabolism are listed in Table 1. Additionally, the B⁺ and B⁻ lines had a non-shared set of genes responding to H₂O₂. In more detail, 3386 DEGs responded to the H₂O₂ uniquely in the B⁺ line, but fewer DEGs (2054) responded uniquely in the B⁻ line.

Assuming that the areas of the Venn diagram (3386 B⁺ and 2054 B⁻ non shared DEGs) may efficiently summarize a part of the variability explained by the fungal-bacterium interaction, GO enrichment was performed on both the subsets (Figs. 4 and 5). The analyses revealed that among the enriched categories of the B⁺ treated vs B⁺ control comparison there was a tendency to upregulation, while the B⁻ treated vs B⁻ control comparison had prevalently down regulated categories. Phosphate and phosphorus metabolic processes were highly represented in both the enriched GO sets, but were up-regulated in the B⁺ and down-regulated in the B⁻ treated spores. Interestingly, response to other organism (GO:0051707) was among the enriched terms only for the B⁺ line upon treatment. Similarly, in the B⁺ treated line two processes associated with DNA and chromatin conformation changes were well represented and up-regulated, while proton transport

340and electron transport chain were down-regulated. It has already been demonstrated that, at least in
341*Candida*, the exposure to H₂O₂ triggers a conformational chromatin change that finely modulates
342the antioxidant responses (da Silva Dantas et al. 2015).

343Two functional categories associated with sporulation (GO:0043937 and GO:0034306) were down-
344regulated in the B- line after treatment. Nitrate assimilation and metabolism were enriched uniquely
345for the B- line, as well as the response to iron ion. An iron transporter was reported to be one of the
346most upregulated transcripts in the B+ control line vs the B- control line (Salvioli et al. 2016.). We
347could thus hypothesize an active role of *CaGg* in regulating the fungal iron homeostasis: in the
348absence of the endobacterium, and in particular under oxidative stress, this process could be
349strongly impaired.

350Processes involved in modification of proteins and macromolecules were also enriched in the B+
351line: since chaperones, heat shock proteins, and ubiquitination/proteasome related genes belong to
352this category, the activation of such pathways suggests that fungal proteins were indeed damaged by
353oxidative stress, and that these processes were essential to maintain the functionality of the cell.

354Finally, some of the transcripts encoding for ROS scavengers illustrated in Table 1, and mainly
355upregulated in the all treated vs all control comparison were down-regulated in the treated B+, when
356compared to the treated B- line. These data were consistent with glutathione S-transferase,
357peroxiredoxin, and glutathione peroxidase (Table 2). This result may be explained by considering
358that the expression of these ROS scavengers is already higher in the B+ control fungus, when
359compared to the B- control (B+ control vs B- control comparison). A similar expression pattern has
360already been described for glutathione S-transferase and peroxiredoxin, which were shown to be
361specifically up-regulated at transcript and protein levels in the B+ line under normal growth
362conditions (Salvioli et al. 2016; Vannini et al. 2016). Thus, given that *G. margarita* B+ efficiently
363elicits the ROS scavengers, it potentially needs a lower activation of these genes when compared to
364the less-efficient B- line upon H₂O₂ treatment.

365

366Glutathione and ascorbate content analysis

367To test whether the response observed at the transcriptional level allows the B+ and B- lines to
368maintain their redox homeostasis, we measured total glutathione and ascorbate, and ascorbate
369analogues, in control and treated fungal lines (Fig. 6). The B- control spores contained significantly
370less GSH than the B+ control spores but had more comparable GSSG content. As a consequence,
371the GSH/GSSG ratio was increased by the presence of the endobacterium (Fig. 6a), confirming
372previous observations that *CaGg* can influence the redox balance of its fungal host (Salvioli et
373al.2016; Vannini et al.2016). The H₂O₂ treatment led to an increase in total glutathione contents for

374both lines. The transcript encoding for glutamate-cysteine ligase (comp33867_c0), an enzyme
375participating in the first steps of glutathione biosynthesis, showed a tendency to up-regulation in the
376all treated vs all control comparison. The same trend was observed while considering the B- and the
377B+ treated lines separately (log₂ fold change: 1.20 for the B- treated line and 0.82 for the B+ treated
378line). Despite the increase in total glutathione, the GSH/GSSG ratio was only slightly changed in
379the treated B+ vs the control B+ samples. By contrast, the increase in glutathione content in the
380treated B- fungus deeply affected the GSH/GSSG ratio, which reached values comparable to those
381of the B+ line. In treated conditions, the maintenance or the increase of GSH/GSSG ratios in B+
382and B- lines, respectively, seem to be due to the increase in the transcript encoding glutathione
383reductase (Table 1).

384In plants, ascorbate plays a critical role in protecting cells against damaging ROS produced by the
385chloroplast during photosynthesis (Wheeler et al. 2015); however, our knowledge of the
386biosynthesis and role of ascorbate in fungi is much more limited. Fungi synthesize a range of
387ascorbate analogues, including 6-deoxy-L-ascorbate, ascorbate glycosides, and the five-carbon
388analogue, D-erythroascorbate (Loewus 1999). However, molecular information on the involved
389genes is limited to yeast, and the description of the pathways leading to the different fungal ASC
390analogues is incomplete (Wheeler et al. 2015). Since at least two enzymes, D-arabinono-1,4-lactone
391oxidase (ALO1) and L-gulonolactone oxidase (GULO), are known to be involved in ascorbate
392biosynthesis in fungi, as a first step we used TBLASTN to look for homologues in the
393transcriptome of *R. irregularis* (Tisserant et al. 2013; Lin et al. 2014) and of *G. margarita* (Salvioli
394et al. 2016). We found that *G. margarita* has distinct transcripts as homologues for the two genes,
395but *R. irregularis* has one homologue that matched both GULO and ALO1 (Table S5). In our
396datasets, the expression of the two *G. margarita* putative homologues did not show an expression
397pattern that can clearly suggest their involvement in the response to hydrogen peroxide.

398The dipyrindyl assay, which measures all the ascorbate-like reductants (Spickett et al. 2000), showed
399that the B- control spores had an higher content of ascorbate or its analogues (hereafter, collectively
400indicated as ASC in the reduced form, and DHA in the oxidized form) than the B+ control spores;
401this indicates that the presence of *CaGg* is involved in the decrease of these compounds (Fig. 6b).
402Treatment with H₂O₂ decreased ASC and DHA levels in the cured lines and had no effect on the
403content of these metabolites in the B+ lines. As for glutathione, H₂O₂ treatment reduced the
404differences between the two lines, which after the treatment had the same ASC and DHA levels.

405

406Discussion

407To verify whether the fungal-endobacterial symbiosis established between *G. margarita* and *CaGg*
408may enhance the fitness of the fungus in the presence of environmental oxidative stress, we treated
409the germinating fungus with a sublethal hydrogen peroxide concentration and performed a detailed
410RNA-seq analysis. Our transcriptomic results indicate that *G. margarita* may efficiently face
411exogenous oxidative stress, and in the presence of its endobacterium upregulates many metabolic
412pathways. However, and contrary to our hypothesis, the cured line responds to H₂O₂ by activating
413the transcription of genes encoding specific ROS scavengers. We confirmed the efficacy of this
414strategy by measuring the glutathione content and redox state of cured and uncured lines, finding
415that, at the end of the treatment, these were the same for both lines.
416We further suggest that glutathione could constitute the main antioxidant molecule for *G.*
417*margarita*, making the role of ascorbate secondary. Indeed, although the ASC and DHA contents in
418the B+ and B- lines subjected to H₂O₂ treatment are lower than in the control B- line, no changes in
419the redox state of these metabolites has been observed, suggesting that in *G. margarita* ascorbate
420and its analogues could play different metabolic roles from the antioxidative one, as already
421suggested for yeast (Spickett et al. 2000).

422

423*Gigaspora margarita* has adaptive mechanisms to face oxidative stress

424

425Hydrogen peroxide has often been used to mimic environmental oxidative stress in many model
426organisms. Our experiments reveal that *G. margarita* is highly sensitive to H₂O₂ treatment, leading
427to a consistent number of DEGs (5458 out of 35029 potential *G. margarita* genes) that respond to
428the treatment, irrespective of the presence of the endobacterium. *G. margarita* therefore is similar to
429yeast and many other filamentous fungi, where H₂O₂ treatment induces a strong detoxifying
430response with the activation of glutathione and thioredoxin metabolism, but can also trigger cellular
431differentiation and development (Breitenbach et al. 2015). Some activated genes in the treated AM
432fungus were expected, such as the genes encoding ROS-detoxifying enzymes listed in Table 1,
433some of which have already been characterized in *R. irregularis* (Tamayo et al. 2016). Other genes
434coding for reducing enzymes linked to glutathione and thioredoxin, and known as the most
435important redox buffers for eukaryotic cells, were up-regulated upon H₂O₂ treatment. The changes
436observed in the expression of several genes coding for iron/heme-binding enzymes and for other
437mitochondrial proteins confirm the role of this organelle in the bacterial-fungal interaction (Salvioli
438et al. 2016). By contrast, the HOG pathway, which is considered a marker for H₂O₂ response in
439yeast (Alonso-Monge et al. 2003), was weakly activated, suggesting that symbiotic filamentous
440fungi have probably elaborated different mechanisms. The upregulation of the gene coding for
441methionine sulfoxide reductase has significance, since methionine-containing proteins directly

participate in oxidative stress responses and methionine itself is often described as a defense against oxidative stress in eukaryotic cells (Luo and Levine 2009). Together with the upregulation of sulfur metabolism and the pentose phosphate pathway, which is essential for sulfur assimilation, being the main source of reducing power (Slekar et al. 1996), we can conclude that the main actors involved in detoxification are at work when *G. margarita* is exposed to H₂O₂ (Fig.7). Given the relatively high number of MAPKs regulated by H₂O₂ in our data set, we also suggest that *G. margarita* strongly relies on this type of signal transduction to face the oxidative stress, as seen for *Candida albicans* (de Dios et al. 2010) and other filamentous fungi (Ding et al. 2015; Jacob et al. 2014). Curiously, a comparable succession of events (activation of pentose phosphate pathway, production of reducing power, recycling of GSSG to GSH, and relief of oxidative damage) has been described when pollinators ingest nectar (Martinez del Rio and Dillon 2017; Levin et al. 2017). This could suggest that the use of the ancient pentose phosphate pathway to avoid oxidative damage may be a conserved strategy for living organisms.

The fungal responses to H₂O₂ change depending on the presence of the endobacterium
The variance partitioning analysis revealed a small effect of the endobacterium in shaping the transcriptome profile of its fungal host in the presence of an oxidative stress; however, we observed that some specific metabolic pathways changed depending on the presence/absence of the endobacterium.

When considering the general pattern of the transcripts, some processes were up-regulated in the B+ line and down-regulated in the B- line. We suggest that, for the B- line, the impairment of several aspects of the primary metabolism (Bonfante and Desirò 2017; Lumini et al. 2007) could impact the way the fungus reacts to stress induced by hydrogen peroxide. A crucial gene category showed an opposite pattern: all the ROS-scavenger genes shown in Table 2, were surprisingly upregulated in the B- line vs the B+ line after H₂O₂ treatment. Since we have previously demonstrated (Salvioli et al. 2016) that the B+ line had constitutively higher expression levels of such ROS scavenging enzymes than the B- line, we reasoned that the line with its endobacteria probably needs less of an increase in the expression of ROS-responsive elements compared with the B- line. Thus, the H₂O₂ treated B- line needs to invest more in eliciting the expression of ROS scavengers, in order to reach an antioxidant status that is comparable to that of the native fungal line.

In contrast to the cured line, the fungus with the endobacteria seems to activate a different strategy. It does not further elicit the transcription of the ROS-scavenger genes following the H₂O₂ exposure, and indeed (under similar conditions) the corresponding bacterial genes were downregulated (Salvioli et al. 2016), suggesting that the *CaGg* does not provide specific help in ROS

476detoxification. However, regulatory systems of *CaGg* like the toxin-antitoxin systems are activated
477by H_2O_2 exposure (Salvioli et al. 2017). Therefore, we suggest that the bacterium provides a
478protection to its fungal host by using more complex mechanisms than the upregulation of ROS-
479scavenger genes. Differently from these genes, many other pathways were upregulated in the
480fungus with the endobacterium. In the treated B+ line processes related to MAPK signal
481transduction (such as phosphorus and phosphate metabolism categories, protein kinase cascade and
482phosphorylation processes) were overall up-regulated, while they were down-regulated in the
483treated B- line. Since the whole MAPK cascade is activated by diverse environmental stresses as a
484powerful actor to control gene expression and conferring cellular stress resistance (Morigasaki et al.
4852013; Hagiwara et al. 2009), we hypothesize that the fungus with its endobacterium may more
486efficiently face oxidative stress, as well as high osmolarity and heat shock. Also DNA
487conformation/packaging GO categories were upregulated in the B+ treated line only. Given that
488 H_2O_2 can act as a stimulus to activate some genomic areas (Breitenbach et al. 2015), the fungal-
489bacterial association could be more efficient in perceiving it.

490
491Our work has demonstrated that an AM fungus actively responds to H_2O_2 used to mimic an
492exogenous source of oxidative stress. Some responses mirror mechanisms already described in
493yeast and filamentous fungi. The presence of the endobacterium leads to more tuned differences: the
494B- line mostly activates the expected ROS-scavenger genes, which are constitutively highly
495expressed in the B+ line, while the B+ line activates many other pathways. These pathways include
496MAPK cascades, which may lead to a more active and tightly regulated response to exogenous
497oxidative stress.

498In conclusion, it seems that the mechanisms for adaptation to oxidative stress in *G. margarita* are
499diverse and differ depending on the presence/absence of the bacterium. The AM fungus with the
500bacteria strongly up-regulate many general processes, which probably also involve the
501endobacterium; the AM fungus without the bacteria show general down-regulation of such
502processes, but specifically activate a set of ROS-related genes. Eventually, the antioxidant status of
503the two fungal lines turns out to be very similar, demonstrating the unexpected capacity of the cured
504line to compensate for its original lower performance. We can speculate that for both the lines H_2O_2
505also acts as a signal, as it does in dried seeds (Tommasi et al. 2001, El-Maarouf-Bouteau and Bailly
5062008) and in fungal conidia also from pathogenic fungi, where changes in the cellular oxidative
507status trigger germination (Breitenbach et al. 2015). Since H_2O_2 is a strong antibacterial molecule,
508the question remains whether *CaGg* is affected by the treatment, also considering that some of the
509key enzymes for glutathione biosynthesis were not found in its genome (Ghignone et al, 2012).

Given the relevance of AM fungi in natural and agricultural ecosystems, we propose that the dataset developed for *G. margarita* may be a starting point for studying environmental adaptation of AM fungi to the oxidative stress that originates from the application of fungicide or herbicide (Yang et al. 2016; Angelova et al. 2005).

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Figure Legends

Fig. 1: (a) Principal Component Analysis (PCA) showing the distance between samples of each condition. Ellipses represent 95% confidence interval around each group; (b) Variance Partitioning Analysis (VPA) executed over the whole dataset, showing hydrogen peroxide treatment as the driving parameter contributing to overall variability.

Fig. 2: GO enrichment analysis representation of the DEGs generated for the all treated vs all control comparison. Each slice of the circle is associated with an enriched GO category. The size of the inner circle slices is proportional to the significance of the related term ($P < 0.05$), and their color indicates if the related category is globally up- or down-regulated. A dot is plotted in the outer circle slices for each gene belonging to a specific category. The color of the dots indicates if the genes are up- (red) or down- (blue) regulated, and their position on the gray spaces is a representation of their log₂ fold change (i.e. genes with the strongest up-regulation are placed on the outer border of the spaces).

Fig. 3: Venn diagram showing the number of DEGs that are in common between the all treated vs all control, B+ treated vs B+ control and B- treated vs B- control comparisons, and genes whose expression changes uniquely for each comparison.

Fig. 4: GO enrichment analysis representation of the DEGs generated for the genes that are DE only for the B- treated vs B- control comparison. Each slice of the circle is associated with an enriched GO category. The size of the inner circle slices is proportional to the significance of the related term ($P < 0.05$), and their color indicates if the related category is globally up- or down-regulated. A dot is plotted in the outer circle slices for each gene belonging to a specific category. The color of the dots indicates if the genes are up- (red) or down- (blue) regulated, and their position on the gray spaces is a representation of their log₂ fold changes (genes with the strongest regulation are placed on the borders of the spaces).

Fig. 5: GO enrichment analysis representation of the DEGs generated for the genes that are DE only for the B+ treated vs B+ control comparison. Each slice of the circle is associated with an enriched GO category. The size of the inner circle slices is proportional to the significance of the related term ($P < 0.05$), and their color indicates if the related category is globally up or down regulated. A dot is plotted in the outer circle slices for each gene belonging to a specific category. The color of the dots indicates if the genes are up- (red) or down- (blue) regulated, and their position on the gray spaces is a representation of their log₂ fold changes (genes with the strongest regulation are placed on the borders of the spaces).

Fig. 6: Glutathione (a) and ascorbate and/or its analogues (b) contents in control and treated spores of *G. margarita*. Bars indicate the total amount of the molecules (GSH and ASC) and the ratios between the reduced and the oxidised form. Values (expressed as picomoles per spore) are the mean

791of three independent biological replicates. Letters indicate whether the differences observed in the
792total amount of GSH and ASC are statistically significant ($p < 0.05$).

793**Fig. 7:** Scheme showing the enzymes activated by *G. margarita* in response to hydrogen peroxide
794treatment and the connection between their activities and ROS detoxification. Methionine rich
795proteins actively participate in ROS scavenging, since methionine reacts with peroxide radicals and
796is oxidized to methionine sulfoxide, being successively reduced by methionine sulfoxide reductase.
797The oxidized enzyme is then restored to the reduced form in a reaction which involves the oxidation
798of thioredoxin, reduced by the enzyme thioredoxin reductase in a NADPH dependent reaction.
799Given its activation in response to hydrogen peroxide, pentose phosphate cycle is suggested as the
800main source of NADPH to counteract oxidative stress in *G. margarita*. NADPH derived from the
801pentose phosphate cycle could also be involved in the assimilation of sulfur, which is the element
802characterizing the functional groups of all the elements reported in the scheme.

803**Table 1:** *G. margarita* ROS detoxifying enzymes and their activation in the all treated vs all control
804comparison.

805**Table 2:** Expression of three *G. margarita* ROS scavengers in different conditions. The transcript
806levels for these genes are higher in the B+ control; therefore, after treatment, the B+ line requires
807less transcriptional activation to achieve the same detoxifying potential as the B-.

808

809Supplemental materials

810**Table S1:** Data for each Illumina single-end library, including the initial yield of raw reads and the
811percentage of reads that survived the trimming with Trimmomatic V.0.36.

812**Table S2:** summary of the differentially expressed genes (DEGs) identified for each comparison
813($FDR < 0.05$; $|\log_2 \text{foldchange}| > 1.5$).

814**Table S3:** list of the most up- and down- regulated genes in each comparison.

815**Table S4:** *G. margarita* homologues for the key genes involved in the yeast HOG1 signaling
816pathway and their expression.

817**Table S5:** *R. irregularis* and *G. margarita* candidates for GULO and ALO1, two enzymes known to
818catalyze the synthesis of ascorbate analogues in fungi. A single *R. irregularis* showed homology to
819both GULO and ALO1, but two distinct candidates were found for *G. margarita*